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Apoptosis by Death Factor

Review

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Introduction

There is an old Japanese saying that "Once we are in the land of the living, we will eventually die." This is true, not only for human beings, but also for the cells that constitute our bodies. By repeated cell division (mitosis) and differentiation, a fertilized egg produces billions of cells to create our bodies. During this process, many surplus or harmful cells are generated, and they must be removed or killed (Jacobson et al., 1997 [this issue of *Cell*]. For example, thymocytes that have failed to rearrange their T cell–receptor gene, or whose T cell receptor may recognize their own tissues, must be eliminated. The magnitude of the cell death is staggering: more than 95% of thymocytes die in the thymus during maturation. Even in adults, senescent cells are removed and replaced by newly generated cells to maintain homeostasis. The cell death that occurs during embryogenesis, metamorphosis, endocrine-dependent tissue atrophy, and normal tissue turnover is "programmed cell death," mediated by a process termed "apoptosis."

Here, I focus on apoptosis controlled by cytokines. Two death factors, Fas ligand (FasL) or tumor necrosis factor (TNF), bind to their receptors and induce apoptosis, killing the cells within hours. In a classic definition of apoptosis, cells die by "suicide;" that is, cells programmed to die would do so autonomously. However, the identification of death factor–receptor pairs that regulate apoptosis indicates that apoptosis can also be controlled by an external killer in some instances.

Death Factor and Receptor

Fas Ligand and the TNF Family

Cytokines are a family of proteins that regulate cellular proliferation and differentiation by binding to their specific receptors on target cells. Cytokines are grouped into at least three subfamilies based on structure, cysteine-knot growth factors, tumor necrosis factor, and helical cytokines. FasL belongs to the TNF family (Suda et al., 1993; Nagata and Golstein, 1995), which includes TNF, lymphotoxin, CD30 ligand, 4-1BB ligand, CD40 ligand, CD27 ligand, and TRAIL (TNF-related apoptosis-inducing ligand). FasL is synthesized as a type II–membrane protein; that is, its N terminus is in the cytoplasm and its C-terminal region extends into the extracellular space. The extracellular region of about 150 amino acids is well conserved (20–25%) among members of the TNF family, while the length and sequence of the cytoplasmic segments differ significantly.

Proteolysis of membrane-associated TNF produces soluble TNF. The proteolysis is mediated by a membrane metalloproteinase (Gearing et al., 1994). Similarly, membrane-bound FasL undergoes metalloproteinase-mediated proteolytic cleavage to generate soluble cytokine (Tanaka et al., 1996). A specific metalloproteinase inhibitor blocks the processing of TNF as well as FasL, suggesting that a similar enzyme cleaves TNF and FasL. Since the CD40 ligand is also cleaved off from the membrane to become soluble, it is likely that all TNF family members are processed to a soluble form. The soluble form of human FasL is functional, but mouse FasL loses its activity when it is cleaved from the membrane. Furthermore, membrane-bound TNF is more active than soluble TNF in activating the type II TNF receptor (Grell et al., 1995). These results may indicate that FasL and TNF work locally via cell–cell interactions under physiological conditions and that the purpose of shedding TNF or FasL is to attenuate the process.

The functional, soluble forms of TNFs as well as human FasL exist as trimers (Tanaka et al., 1997). It has not yet been demonstrated whether membrane-bound TNF or FasL are trimers. However, lymphotoxin β, a member of the TNF family, consists of a heterotrimer of one α (lymphotoxin-α, or TNFβ) and two β chains (lymphotoxin-β) on the membrane (Androlewicz et al., 1992), suggesting that membrane-bound TNF and FasL have the potential to form trimeric structures. X-ray diffraction analyses of TNFα and TNFβ have indicated that each monomer forms an elongated, antiparallel β-pleated sheet sandwich with a jelly roll topology (Jones et al., 1989). Amino acids conserved among members of the TNF family are mainly within the β strands. Computer-assisted modeling of FasL based on the amino acid sequence suggests that FasL has a similar tertiary structure to TNFα and TNFβ.

The Fas and TNF Receptor Family

Fas (also known as APO-1 or CD95), the receptor for FasL, is a type I–membrane protein (Itoh et al., 1991; Oehm et al., 1992) and a member of the TNF receptor (TNFR) family, which includes two TNFRs (TNFR1 and TNFR2), the receptor for lymphotoxin-β, the NGF receptor (p75), CD40, CD27, and CD30 (Nagata and Golstein, 1995). This family is still growing, and three new members have recently been identified. They are human DR-3 (death receptor-3)/Wsl-1 (Chinnaiyan et al., 1996; Kiston et al., 1996), human HVEM (herpes virus early mediator) (Montogomery et al., 1996), and chicken CAR1 (cytopathic avian leukosis-sarcoma virus receptor) (Brojatsch et al., 1996). The extracellular region of the TNF receptor family members carries 2–6 repeats of a cysteine-rich subdomain that has about 25% similarity among various members. In contrast, the cytoplasmic regions have little similarity among the members, except for Fas, TNFR1, DR-3/Wsl-1, and CAR1, as discussed below.

TNF induces apoptosis and activates the transcription factor NF-κB. It can also stimulate the proliferation of thymocytes. Although both TNFR1 and TNFR2 can

transduce the signal for apoptosis and NF-κB activation, TNFR1 is responsible for these signals in most cases (Vandenabeele et al., 1995). On the other hand, TNFR2 but not TNFR1 is responsible for the TNF-induced proliferation signal in thymocytes. Binding of FasL to Fas or cross-linking Fas with agonistic antibodies (IgM class anti-Fas antibody, or IgG3 class anti-APO1 antibody) induces apoptosis in Fas-bearing cells (Trauth et al., 1989; Yonehara et al., 1989; Itoh et al., 1991). Most other receptors in the TNF receptor family transduce activation or stimulatory signals, although some of them, such as CD40 and CD30, may also have the ability to inhibit growth, probably causing apoptosis. The presence of a homologous domain (about 80 amino acids) in the cytoplasmic regions of Fas and TNFR1 suggested that this region is responsible for transducing the death signal. In fact, subsequent mutational analyses in Fas and TNFR1 indicated that this is the case, and this domain has been designated a death domain (Itoh and Nagata, 1993; Tartaglia et al., 1993). DR-3/Wsl-1 also carries a death domain and has the potential to transduce an apoptotic signal, as well as to activate NF-κB (Chinnaiyan et al., 1996; Kiston et al., 1996). CAR1, which also contains a death domain, has been shown to cause apoptosis in chicken cells when it is cross-linked by the envelope protein of ALSV (Brojatsch et al., 1996).

The death domain has a tendency to self-aggregate, and the tertiary structure of the Fas death domain, revealed by heteronuclear multidimensional NMR spectroscopy, shows that the death domain is a novel protein fold consisting of six antiparallel, amphipathic α helices (Huang et al., 1996). Many charged amino acids are present on the surface, which is probably responsible for mediating the interactions between death domains described below.

Signal for Apoptosis

Cascade Leading to ICE

Binding of ligand to a tyrosine kinase receptor, such as PDGF or EGF receptor, induces dimerization of the receptor and activates the intrinsic kinase activity in the cytoplasmic domain. The receptors for hematopoietic growth factors such as colony-stimulating factor and for interferons do not contain kinase domains in their cytoplasmic regions. Instead, the ligand-induced dimerization recruits a kinase(s) to the receptor and activates it, which then results in transduction of the proliferation and/or differentiation signals. In the case of Fas or TNFR1, however, dimerization with a divalent anti-Fas or TNFR1 monoclonal antibody is not sufficient to activate these receptors. Fas and TNFR1 must be oligomerized to be activated; that is, IgM class anti-Fas monoclonal antibody or IgG3 class anti-APO1 antibody that possess a tendency to aggregate function as potent agonists (Trauth et al., 1989; Yonehara et al., 1989). X-ray diffraction analysis of the TNFβ–TNF receptor complex has indicated that a TNFβ trimer makes a complex with three molecules of the extracellular region of the TNF receptor (Banner et al., 1993), suggesting that TNF induces trimerization of the receptor. The similarity between the structures of FasL and TNF and between Fas and the TNF receptors suggests that FasL also induces trimerization of Fas and that the trimerized cytoplasmic region then transduces the signal.

Fas- and TNFR1-mediated apoptosis occur in the presence of inhibitors of either RNA or protein synthesis (Yonehara et al., 1989; Itoh et al., 1991). Even enucleated cells undergo apoptosis upon Fas activation (Schulze-Osthoff et al., 1994), suggesting that all of the components necessary for apoptotic signal transduction are present and that Fas activation simply triggers this machinery. To dissect the signal-transducing machinery for Fas- and TNFR1-mediated apoptosis, two approaches have been used. In one approach, several groups have identified a molecule(s) that binds to the cytoplasmic region of Fas or TNFR1, while in the other, information gained from studying apoptosis in the nematode, C. elegans, was applied to the Fas and TNF system.

Utilization of the yeast two-hybrid system with the Fas cytoplasmic region as bait led to the identification of a molecule called FADD (Fas-associating protein with death domain) or MORT1, which contains a death domain at its C terminus (Boldin et al., 1995; Chinnaiyan et al., 1995). FADD/MORT1 is recruited to Fas upon its activation (Kischkel et al., 1995) and binds to Fas via interactions between the death domains. The N-terminal region (termed the death effector domain [DED] or MORT1 domain) is responsible for downstream signal transduction. A similar death domain–containing protein (TRADD, TNFR1-associated death domain protein) binds to TNFR1 (Hsu et al., 1995). But unlike FADD/MORT1, TRADD does not carry a death effector domain, and its death domain is responsible for mediating apoptosis. This apparent discrepancy between FADD/MORT1 and TRADD is resolved by the finding that TRADD binds to FADD/MORT1 via interactions between their death domains (Hsu et al., 1996b). These results suggest that Fas and TNFR1 use FADD as a common signal transducer and share the signaling machinery downstream of FADD/MORT1 (Figure 1). In addition to this pathway, TNFR1 has another pathway leading to apoptosis. RIP (receptor interacting protein), originally identified as a Fas-binding protein, preferentially binds to TRADD (Hsu et al., 1996a). RIP is a serine/threonine kinase containing a death domain and binds to TRADD via interactions between their death domains. RIP induces apoptosis when overexpressed. The death domain of RIP, but not its kinase domain, is responsible for transduction of the death signal, indicating that RIP does not possess a death effector domain, but rather another downstream effector molecule may be recruited through the death domain of RIP (see below) (Figure 1).

To find the signaling molecule downstream of FADD/MORT1, Wallach and his associates again used the yeast two-hybrid system, using the N-terminal DED/MORT1 domain of FADD/MORT1 as bait (Boldin et al., 1996). At the same time, a collaborative group, led by Dixit and Peter, continued the biochemical characterization of molecules recruited to the activated Fas receptor (Muzio et al., 1996). Both groups identified the same molecule, which was originally termed FLICE (FADD-like ICE) or MACH (MORT1-associated CED-3 homologue) and is now designated caspase-8 (Alnemri et al., 1996) (Table 1). Caspase-8 carries two DED/MORT1 domains at the N-terminal region, through which it binds FADD/MORT1. The C-terminal region of caspase-8 is related to ICE family members, more specifically, to

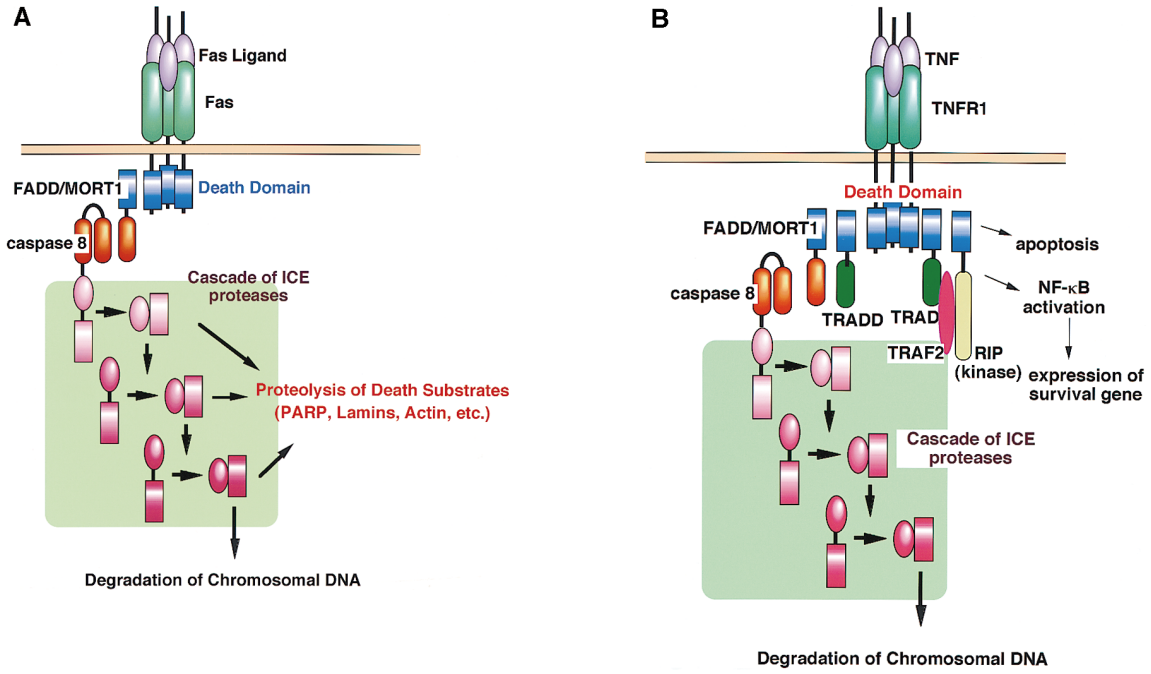

Figure 1. Models for Apoptosis Signaling by Death Factors

(A) Fas-induced apoptosis. Binding of FasL to Fas induces trimerization of the Fas receptor, which recruits caspase-8 (FLICE/MACH) via an adaptor, FADD/MORT1. The oligomerization of FLICE may result in self-activation of proteolytic activity and trigger the ICE protease cascade. The activated ICE members can cleave various substrates, such as poly(ADP) ribose polymerase (PARP), lamin, rho-GDI, and actin, and cause morphological changes to the cells and nuclei.

(B) TNF-induced apoptosis. TNF binds to TNFR1, and the trimerized receptor recruits TRADD via interactions between death domains. The death domain of TRADD then recruits FADD/MORT1 in one pathway to activate caspase-8. In another pathway, RIP binds to TRADD and transduces an apoptotic signal through the death domain. In addition, RIP together with TRAF2 activates NF-κB, which may induce the expression of survival genes. The role of the kinase activity of RIP is currently unknown.

members of the caspase-3 (CPP32) subfamily, and recombinant caspase-8 preferentially cleaves caspase-3 substrates over caspase-1 (ICE) substrates (Boldin et al., 1996).

Figure 1 presents the current model for Fas- and TNFR1-mediated apoptosis. Binding of a trimeric FasL to Fas induces trimerization of Fas, and FADD/MORT1 binds to the trimerized Fas cytoplasmic region through the interaction of the respective death domains. Caspase-8 is then recruited to FADD/MORT1 through binding of the DED domains, which in turn may induce self-activation of the protease domain. One apoptotic pathway from TNFR1 uses caspase-8 pathway through the interaction of TRADD with FADD/MORT1. TRADD additionally recruits RIP, which may trigger a second apoptotic pathway. The recently identified DR-3/Wsl-1 receptor is more similar to TNFR1 than to Fas. That is, DR-3 binds TRADD, which then recruits FADD and RIP (Chinnaiyan et al., 1996; Kiston et al., 1996). The apoptotic signaling pathway downstream of RIP is currently unknown. However, another death domain–containing adaptor, termed RAIDD (RIP-associated Ich-1/CED-3

Table 1. Human ICE Protease Superfamily

Proteases	Alternative Names	Recognition Sequence	Substrates
caspase-1	ICE	YVAD	pro-IL1β, pro-caspase 3 and 4
caspase-4	ICErel-II, TX, ICH-2		
caspase-5	ICErel-III, TY		
caspase-2	ICH-1		PARP
caspase-9	ICE-LAP6		PARP
caspase-3	CPP32, Yama, apopain	DEVD	PARP, DNA-PK, SRE/BP, rho-GDI
caspase-6	Mch2	VEID	lamin A
caspase-7	Mch3, ICE-LAP3, CMH-1		PARP, pro-caspase 6
caspase-8	FLICE, MACH, Mch5		
caspase-9	ICE-LAP6, Mch6		PARP
caspase-10	Mch4		

The caspase family members can be divided into three subfamilies: caspase-1 (ICE), caspase-2 (ICH-1), and caspase-3 (CPP32), according to Alnemiri et al. (1996).

homologous protein with a death domain) has recently been identified (Duan and Dixit, 1997). RAIDD binds RIP through its death domain and recruits caspase-2 (Ich-1) to RIP. Although an involvment of RAIDD in the TNFR1 or DR3/Wsl-1-mediated apoptotic pathway has not yet been demonstrated, it is possible that RAIDD plays a role in transducing an apoptotic signal from one of the death receptors.

The signal from Fas seems to be restricted to apoptosis, whereas other members of the TNF receptor family including TNFR1 activate NF-κB. NF-κB activation by TNF receptor family members is mediated by TRAF (TNF receptor–associated factor) family (Rothe et al., 1994). So far, five members have been identified in this family, and all contain a TRAF domain of about 230 amino acids. Among members of this family, TRAF2 binds directly to TNFR2 and CD30 and indirectly to TNFR1 through TRADD and RIP. A dominant-negative TRAF2 blocks TNF-induced NF-κB activation, but not apoptosis (Liu et al., 1996). Instead, blocking NF-κB activation with the dominant-negative TRAF2 potentiates the cytotoxic activity of TNF in various cell types, suggesting that NF-κB activation leads to the expression of a protein(s) that inhibits TNF-induced cytotoxicity. NF-κB consists of two subunits (p50 and p65) and exists in a complex with IκB in resting cells. The signal from TRAF2 results in phosphorylation of IκB and subsequent degradation by the proteosome. NF-κB, thus released from IκB, enters the nucleus and activates various genes carrying the NF-κB response element. Cells lacking one component of NF-κB (65 kDa) or expressing IκB mutants that cannot be phosphorylated are more sensitive to TNF-induced cytotoxicity, confirming that one of the target genes for NF-κB is a gene encoding a survival factor (Beg and Baltimore, 1996; Liu et al., 1996; Van Antwerp et al., 1996; Wang et al., 1996). These results are in good agreement with the fact that Fas, which cannot activate NF-κB, mediates a stronger apoptotic signal than TNFR1, which can activate NF-κB. The cytotoxicity of TNF can be potentiated by cycloheximide or actinomycin D, which is probably due to the inhibition of the NF-κB-induced gene expression.

ICE Protease Cascade

Genetic analysis of programmed cell death in C. elegans has revealed a number of gene products that regulate the cell death process (Ellis et al., 1991). Among them, the CED-3 product is required for cell death, and molecular cloning of the *ced-3* gene revealed it to be a homologue of mammalian ICE (interleukin-1β converting enzyme) (Yuan et al., 1993), which converts the IL-1β precursor to the mature form. ICE is a cysteine protease consisting of two large (p17) and two small (p10) subunits, which are generated by proteolytic cleavage of the ICE precursor (a zymogen). Cross-hybridization with ICE cDNA and a search of the human genome database revealed at least 10 ICE homologues (see Table 1), which are divided into three subgroups (ICE-like, CPP32-like, and Ich1-like proteases), based on their sequence homology (Alnemri et al., 1996). All of these cause apoptosis when overexpressed in cells. They appear to be cysteine proteases, containing conserved sequences for substrate binding and catalysis; they cleave their substrates after aspartic acid. Therefore, they are now designated as caspases (cysteine aspases) (Table 1) (Alnemri et al., 1996). So far, recognition sequences for three ICE family members have been identified. That is, caspase-1 (ICE) recognizes the sequence Tyr–Val–Ala–Asp (YVAD) in the proform of IL-1β, caspase-3 (CPP32/Yama/apopain) recognizes Asp–Glu–Val-Asp (DEVD) and cleaves poly(ADP-ribose) polymerase, and caspase-6 (Mch2) recognizes Val–Glu–Ile–Asp (VEID) and cleaves lamin (Nicholson et al., 1995; Takahashi et al., 1996). However, it is uncertain whether each ICE family member has a specific substrate for mediating apoptosis, or if some members of the subfamily are redundant, cleaving the same substrates. In this regard, it is noteworthy that caspase-1-null mice do not show any phenotype in programmed cell death (Li et al., 1995), while the mice lacking caspase-3 show hyperplasia and disorganized cell development in the brain (Kuida et al., 1996). These results suggest that caspase-1 is redundant in all cell types, while caspase-3 plays a major role in apoptosis in some cells of the brain.

Using what was known about the specific recognition sequences of the ICE proteases, specific competitive inhibitors and fluorescent substrates for caspase-1 and -3 have been designed (Thornberry et al., 1992). In addition, several proteins encoded by viral genes are known to inhibit members of the ICE family. These include *crmA*, a cytokine response–modifier gene encoded by cowpox virus, and p35, coded for by Baculovirus. These viral proteins seem to inhibit protease activity by forming a stable complex. p35 has a broader specificity for ICE family members than *crmA*. That is, *crmA* preferentially inhibits caspase-1 over caspase-3, while p35 inhibits both caspase-1 and -3 equally well.

Inhibitors of caspase-1 or -3 block Fas- and TNF-induced apoptosis, which suggests that both caspase-1- and caspase-3-like proteases are involved in Fas- and TNFR1- mediated apoptosis (Enari et al., 1995b; Los et al., 1995; Tewari and Dixit, 1995; Enari et al., 1996). Monitoring the protease activity with specific fluorescent substrates for caspase-1 and -3 demonstrates that a caspase-1-like protease is transiently activated, whereas the activation of a caspase-3-like protease gradually increases during Fas-induced apoptosis (Enari et al., 1996). A similar sequential activation of caspase-1- and caspase-3-like proteases was also found in vivo. When agonistic anti-Fas antibody was administered to mice, the livers were damaged (Ogasawara et al., 1993). As the damage proceeded, caspase-1-like activity was detected in the liver, followed by the gradual activation of a caspase-3-like protease (Rodriguez et al., 1996a). The activation of the caspase-3-like protease is dependent on the activation of a caspase-1-like protease (Enari et al., 1996), indicating that these proteases are sequentially activated. This sequential activation can also be seen in a cell-free system. That is, cell lysate from Fas-activated, but not from nonactivated cells, induced apoptotic morphological changes in intact nuclei (Enari et al., 1995a). However, when the cell lysates from growing, nonapoptotic cells were supplemented with recombinant caspase-1 or -3, the lysates induced apoptosis. This caspase-1-induced apoptosis was inhibited, not only by an inhibitor of caspase-1, but also by the inhibitor of caspase-3 (Enari et

al., 1996), confirming the sequential activation of caspase-1- and caspase-3-like proteases. It is likely that other members of the ICE family are also activated in the cascade, cleaving their "death substrates" such as lamin, actin, poly(ADP)ribose polymerase, rho-GDI, SREBP, and DNA-dependent protein kinase, to cause the apoptotic morphological changes observed on cells and nuclei, as well as chromosomal DNA degradation.

As discussed above, Fas engagement recruits caspase-8 to the Fas receptor complex. How can this result be integrated into the model of sequential ICE protease activation? Here, I suggest two models. In the first model, the oligomerization of caspase-8 through the interaction with FADD/MORT1 leads to its autocatalytic activation, which then triggers the protease cascade by cleaving the caspase-1-like protease zymogen. In the second model, oligomerization does not activate caspase-8, but a caspase-1-like protease activates the oligomerized caspase-8, which then sequentially activates other members of the ICE family. In addition to ICE family proteases, other proteases such as cathepsin D aspartic protease and the serine protease AP24 (apoptosis protease 24) may be involved in Fas- and TNFR1-induced apoptosis. To understand how these proteases may be involved in the apoptotic process, it will be necessary to biochemically characterize the purified or recombinant proteins and determine their specific substrates.

The Bcl-2 Family

ced-9, a homologue of the mammalian protooncogene *Bcl-2*, prevents programmed cell death in C. elegans (Hengartner and Horvitz, 1994). Similarly, overexpression of Bcl-2 blocks apoptosis of mammalian cells that is triggered by a number of different stimuli such as factor deprivation, irradiation, c-*myc*, or anti-cancer drugs. A number of CED-9/Bcl-2 family members have been identified in mammals: Bcl-2, Bcl-xL, Bcl-w, and Mcl-1 inhibit apoptosis, whereas others, such as Bax, Bik, Bak, Bad, and Bcl-xs, activate apoptosis. The various Bcl-2 family members can dimerize with one another, with one monomer antagonizing or enhancing the function of the other. In this way, the ratio of inhibitors to activators in a cell may determine the propensity of the cell to undergo apoptosis (Yang and Korsmeyer, 1996). For example, if either *bcl-x* (Motoyama et al., 1995) or *bcl-2* (Veis et al., 1993) is disrupted in mice, the animals die as embryos or postnatally, respectively, as the result of excessive programmed cell death in particular organs. Conversely, if *bax* is disrupted, some normal programmed cell death fails to occur (Knudson et al., 1995). Another attractive mechanism to regulate dimerization of Bcl-2 family members is phosphorylation (Gajewski and Thompson, 1996). For example, Bad, a proapoptotic member of the Bcl-2 family, is phosphorylated by a putative kinase that can be activated by growth factor engagement. The phosphorylated Bad loses the ability to bind Bcl-xL. Instead, it binds to 14-3-3, a protein that can interact with several signaling enzymes. The Bcl-xL dissociated from Bad now can execute its antiapoptotic function (Zha et al., 1996).

How does Bcl-2 or Bcl-xL inhibit apoptosis? Genetic studies of *ced-9*, *ced-4*, and *ced-3* mutants in C. elegans indicate that *ced-9* controls programmed cell death upstream of *ced-4* and *ced-3* (Shaham and Horvitz, 1996). However, little is known about the biochemical mechanism whereby CED-9/Bcl-2 and their family members inhibit apoptosis. Bcl-2 and Bcl-x are localized to outer mitochondrial membranes and endoplasmic reticulum as well as nuclear membranes. The tertiary structure of Bcl-xL has been determined by X-ray and NMR analyses (Muchmore et al., 1996). It consists of two central, hydrophobic α helices, which are similar to the pore-forming bacteria toxins such as diphtheria toxin and the colicins, suggesting that Bcl-xL also generates pores in the membrane. When mitochondria are damaged by an agent that causes permeability transition, nuclear apoptosis is induced (Zamzami et al., 1996). This permeability transition of mitochondrial membrane, and thus nuclear apoptosis, is blocked by Bcl-2, suggesting that the membrane pores in the mitochondria, generated by the Bcl-2 family members, play an important role in apoptosis, at least in this system.

Bcl-2 and Bcl-xL can also inhibit Fas-mediated apoptosis in vitro as well as in vivo (Itoh et al., 1993; Boise et al., 1995; Rodriguez et al., 1996b). Fas activation damages mitochondrial function, but the damage is inhibited by ICE protease inhibitors (Krippner et al., 1996). These results suggest that the mitochondrial damage is downstream of the ICE protease cascade in Fas-induced apoptosis and is probably a secondary effect. Thus, it is not clear how Bcl-2/Bcl-xL located in mitochondria can modulate the Fas-induced apoptotic signaling pathway that seems to take place in the cytoplasm. One possible mechanism is that the damage of mitochondria by ICE protease may amplify the signal by releasing apoptosis-inducing molecules (Krippner et al., 1996; Zamzami et al., 1996).

Other Regulators in the Signaling Pathway

Ceramide, generated by sphingomyelinases, increases during Fas- or TNFR1-mediated apoptosis, and ceramide itself can induce cell death (Spiegel et al., 1996). Since ceramide activates the ras/MAP kinase pathway, it was postulated that activated ras is responsible for apoptotic cell death. However, the recent observation that generation of ceramide and activation of JNK during Fas activation is blocked by ICE protease inhibitors suggests that the production of ceramide occurs downstream of the ICE protease cascade (Gamen et al., 1996; Lenczowski et al., 1997). An increase in ceramide during Fas activation is likely to be one of the changes that accompanies apoptosis and is unlikely to be a mediator of apoptosis. Many other proteins have been suggested as regulators of Fas-mediated apoptosis. For example, c-*abl* tyrosine kinase, FAP tyrosine phosphatase, and small stress proteins (HSP24) inhibit the process, whereas the Fas-associated proteins of $p59^{fyn}$ kinase and FAF seem to augment apoptotic signal induced by Fas. How these proteins regulate the process is currently unknown.

Physiological and Pathological Roles of Fas

Down-Regulation of the Immune Reaction

Apoptosis occurs in various processes in mammalian life (Jacobson et al., 1997). What kinds of apoptosis are regulated by the Fas system? Fas is ubiquitously expressed in various tissues with abundant expression

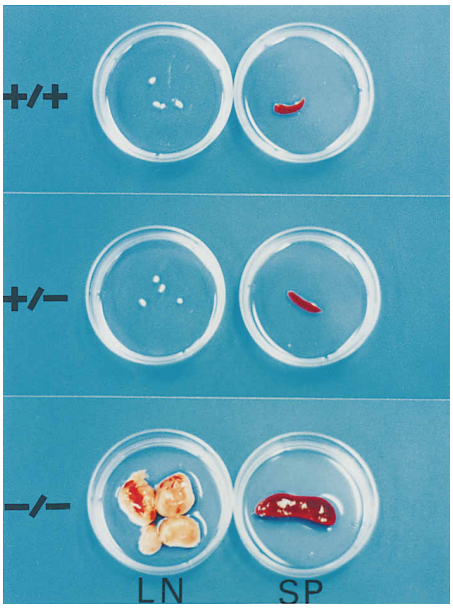

Figure 2. When Apoptosis Fails

Wild-type (+/+) and Fas-null (−/−) mice were killed at 16 weeks of age, and their lymph nodes (LN) and spleen (SP) are shown (from Adachi et al., 1995).

in the thymus, liver, heart, and kidney. On the other hand, FasL is predominantly expressed in activated T lymphocytes and Natural Killer (NK) cells, although it is also expressed constitutively in the tissues of the "immune-privilege sites" such as the testis and eye, as described below. The mouse mutations, *lpr* (lymphoproliferation) and *gld* (generalized lymphoproliferative disease), are spontaneous recessive mutations (Cohen and Eisenberg, 1991). Mice carrying homozygous mutations in *lpr* or *gld* develop lymphadenopathy and splenomegaly by accumulating $CD4^{-}CD8^{-}$ cells of T cell origin, and some strains of mice develop autoimmune diseases. Genetic and molecular analyses of *lpr* and *gld* mutations showed that they are loss-of-function mutations in the Fas and FasL genes, respectively (Watanabe-Fukunaga et al., 1992; Takahashi et al., 1994). The Fas-null mice, established by gene targeting (Adachi et al., 1995), also show lymphadenopathy and splenomegaly (Figure 2), which is much more pronounced than in mice carrying the leaky *lpr* mutation. Furthermore, when Fas was expressed in the lymphocytes of *lpr* mice as a transgene, the lymphoproliferative phenotype was rescued (Wu et al., 1994), confirming that Fas plays a role in the programmed cell death of T lymphocytes.

T lymphocytes, which are responsible for removing virally infected and cancerous cells, die at various stages of their development. Most immature T cells are useless (incorrect rearrangement of the T cell receptor) or potentially detrimental (self-reactive) to the organism. More than 95% of thymocytes that immigrate into the thymus are eliminated by positive and negative selection during their development. In the periphery, mature T cells that recognize self antigens are also deleted (peripheral clonal deletion). When mature T cells encounter target cells, they are activated to proliferate. However, after the activated T cells accomplish their task, they must be removed to avoid accumulation. Mature T cells from *lpr* or *gld* mice do not die after activation, and activated cells accumulate in the lymph nodes and spleens of these mice. When T cell hybridomas are activated in the presence of a Fas-neutralizing molecule, they do not die. These results indicate that Fas is involved in activation-induced suicide of T cells, i.e., in down-regulation of the immune reaction (Figure 3A) (Nagata and Golstein, 1995). Peripheral clonal deletion may also be mediated by the Fas system, because the cells to be deleted in this process are activated by interactions with cells expressing self antigens. However, thymic clonal deletion is apparently normal in mice lacking the functional Fas system (*lpr*-, *gld*-, or Fas-null mice) (Singer and Abbas, 1994), even though thymocytes abundantly express Fas and are sensitive to Fas-induced apoptosis. These results suggest that Fas is not involved in the deletion process in the thymus, although one cannot rule out the possibility that this process is mediated by redundant mechanisms.

In addition to T cells, the Fas-deficient mice accumulate B cells and have elevated levels of immunoglobulins of various classes that include anti-ssDNA and anti-dsDNA antibodies (Cohen and Eisenberg, 1991), suggesting an involvement of the Fas system in the deletion of activated or autoreactive B lymphocytes. In fact, immunization of mice with antigens rapidly induces Fas expression in germinal centers. Furthermore, the activation of naive B cells through CD40 sensitizes them to Fas-mediated apoptosis, while their costimulation through CD40 and Ig receptor makes them resistant (Rothstein et al., 1995). Although these results suggest that FasL-expressing T cells kill the Fas-expressing activated B cells, the precise mechanism and physiological role of Fas in the deletion of B cells remains to be studied.

Children carrying a defect in the Fas gene have also been identified (Fisher et al., 1995; Rieux-Laucat et al., 1995). Most of these patients carry a heterozygous mutation in the Fas gene. The affected Fas protein seems to work in a dominant-negative fashion, and T cells from the patients do not die upon activation. The patients show phenotypes (ALPS, autoimmune lymphoproliferative syndrome) that are remarkably similar to those of *lpr* mice, including lymphadenopathy, splenomegaly, and hypergammaglobulinemia. Some patients show autoimmune diseases such as hemolytic anemia, thrombocytopenia, and neutropenia by producing autoantibodies against red blood cells and platelets. On the other hand, the fathers or mothers of the patients, who also carry the heterozygous mutation of the Fas gene, do not show an abnormal phenotype, suggesting that the patients carry mutations in other complementing genes. Alternatively, Fas could be required only for the perinatal period, and the parents may also have had a similar phenotype in childhood that was rescued later, since the heterozygous mutation is leaky.

Effector of Cytotoxic T Lymphocytes and Natural Killer Cells

Cytotoxic T lymphocytes (CTL) recognize and kill cells infected by viruses or bacteria, while NK cells kill cancerous cells. The professional CTL are $CD8^{+}$ T cells, but

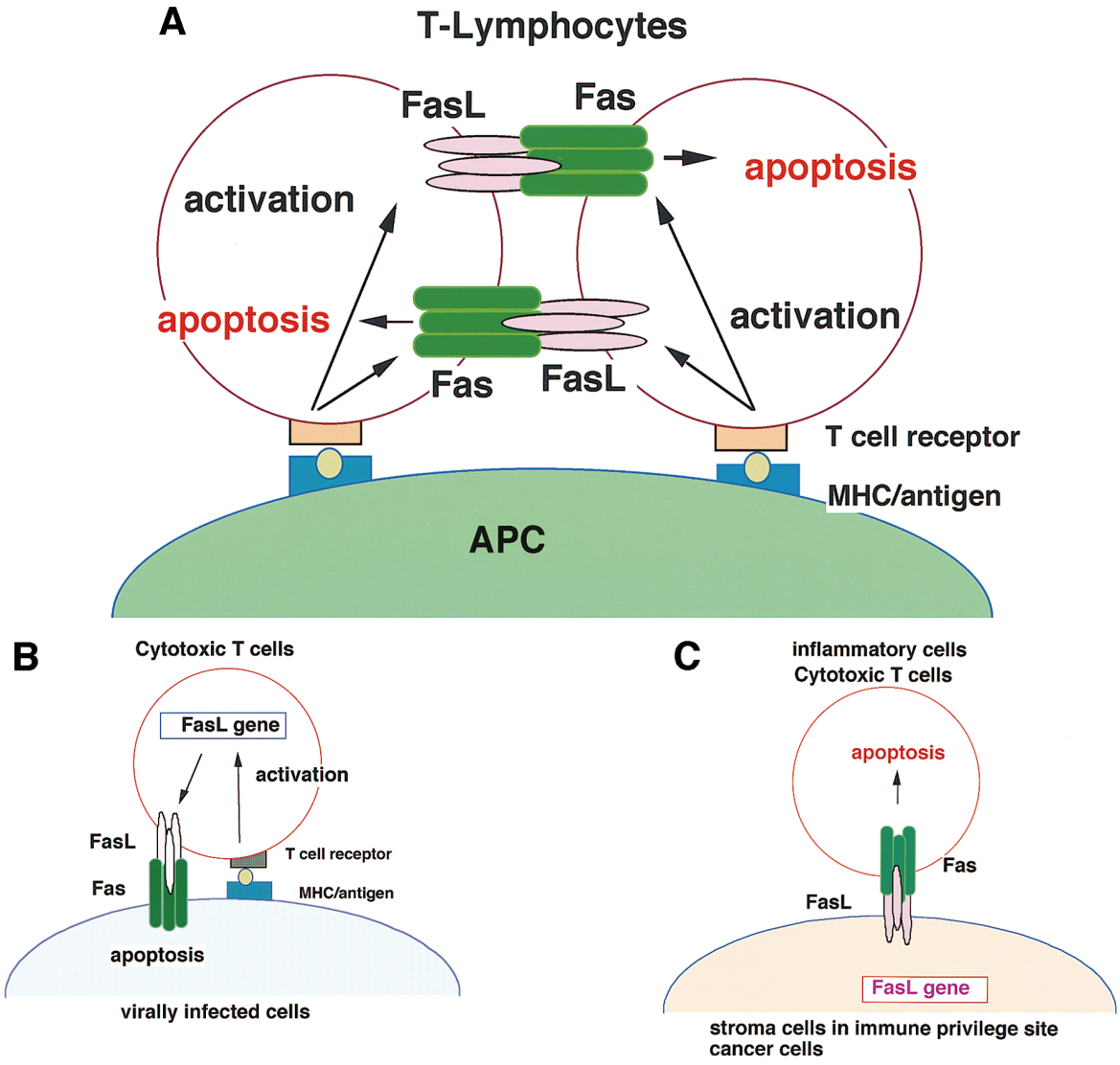

Figure 3. Three Types of Killing by the Fas and FasL System

(A) Activation-induced suicide of T cells. Mature T lymphocytes are activated by T cell–receptor interaction with antigen-presenting cells. The activated T cells express FasL, which binds to the Fas-expressing activated T cells to induce apoptosis.

(B) CTL-mediated killing of target cells. Virally infected cells present viral antigen as a complex with MHC. The cytotoxic T cells recognize the antigen and become activated, leading to the expression of FasL. FasL then binds to Fas on the target cells to induce apoptosis.

(C) Killing of inflammatory cells in immune privilege sites and killing of CTL by tumor cells. Stromal cells in the immune privilege sites such as the eye and testis and some tumor cells constitutively express FasL. When activated T cells or neutrophils enter an immune privileged site, FasL binds to Fas on these cells and kills them to prevent inflammation. Similarly, when CTL or NK cells approach tumor cells, the tumor cells counterattack these cells to escape from the immune destruction.

Th1-type $CD4^+$ T cells also show cytotoxicity. How these CTL and NK cells kill target cells was under debate for a long time, because a well-known perforin/granzyme-based mechanism could not account for all of the examples of CTL killing. However, the identification of FasL as a cytotoxic molecule expressed by activated T cells resolved this problem. Studies with mice deficient in either perforin/granzyme or FasL indicated that the perforin/granzyme and FasL systems are major pathways for CTL-mediated cytotoxicity (Nagata and Golstein, 1995). Activation of CTLs through T cell–receptor interaction with viral antigens induces the expression of the FasL gene. The FasL expressed on the surface of the effector cells binds to Fas on the target cell and causes apoptosis by activating caspases, as described above (Figure 3B). A similar activation of CTLs through T cell receptor would release perforins and granzymes that were stored in granules. It is believed that perforin makes pores in the plasma membrane of the target cells, through which granzymes are introduced. One of the granzymes (granzyme B) is a serine protease aspase, which activates some of the caspase family members by proteolysis (Darmon et al., 1995). Thus, although perforin/granzyme and FasL can independently trigger the cell death program, the processes leading to apoptosis are similar in both cases. The $CD8^+$ T cells and NK cells

use both the perforin/granzyme and FasL/Fas pathways, whereas the Th1-type CD4 T cells preferentially use the FasL system. Whether particular $CD8^+$ T cells and NK cells have any preference for using the perforin/granzyme or FasL/Fas system on specific target cells remains to be studied. Furthermore, CTLs in mice deficient in both the perforin and FasL systems show some residual cytotoxicity in long-term assays (Braun et al., 1996), suggesting that yet another death factor(s), perhaps TNF or TRAIL, functions as a CTL effector under these conditions.

Immune Privilege

Cellular immune response reactions and their associated inflammatory responses can cause nonspecific damage to nearby tissues. Although most organs can tolerate such inflammation, some, such as the eye and testis, cannot. These organs, therefore, have a mechanism to protect themselves against dangerous and unwanted immune reactions. These organs are called "immune privilege sites" and are able to support allogenic and xenogeneic tissue transplants. Initially, it was thought that immune privilege is maintained by preventing the activated cells from entering the organs. However, another attractive mechanism has recently been proposed (Bellgrau et al., 1995; Griffith et al., 1995). That is, although the activated inflammatory cells can enter these organs, they are immediately killed by FasL expressed in the organs (Figure 3C). The constitutive expression of functional FasL has been found in the corneal epithelium and endothelium, iris, and ciliary cells of the eye, as well as in the Sertoli cells of the testis. When the eyes of wild-type mice were infected with herpes simplex virus (HSV-1), very few inflammatory cells were found associated with the retina, while massive inflammation was observed in the retina of *gld* mice, which have a defect in FasL. Furthermore, while testes expressing functional FasL survived when transplanted under the kidney capsule of allogenic animals, testis grafts from *gld* mice were rejected. These findings indicate that FasL accounts for at least part of the immune-privileged nature of the eye and testis and suggest a use for FasL as an immunosuppressive agent to target activated effector cells in transplantation. In fact, when islets of Langerhans were cotransplanted with syngeneic myoblasts expressing functional FasL, they were protected from immune rejection and were able to maintain normoglycemia for a substantial period in a mouse model system for diabetes (Lau et al., 1996). Similarly, several groups have recently found that some tumor cells become resistant to Fas-induced apoptosis and constitutively express FasL (Hahne et al., 1996; Strand et al., 1996). FasL expressed on tumor cells then counterattacks CTL and NK cells by binding Fas on their surfaces to cause apoptosis. This mechanism may also account for the ability of tumor cells to evade immune destruction.

A Double-Edged Sword

As long as death factors are appropriately expressed, they will be useful in maintaining homeostasis. However, if the system under- or over-functions, it will have deleterious effects. Loss of function causes hyperplasia, such as lymphoproliferation. The lymphocytes accumulated in patients carrying the heterozygous mutation in the Fas gene are not tumorigenic. However, the families of these patients sometimes have histories of Hodgkin's lymphoma (Fisher et al., 1995). The abnormal survival of the lymphocytes may allow the cells to accumulate mutations that lead to malignancy. Genes for death factors and their receptors, such as FasL and Fas, may therefore be regarded as tumor suppressor genes. On the other hand, when the system overfunctions, it causes tissue destruction and kills the animals. When an agonistic anti-Fas antibody or recombinant FasL was injected into mice to activate the Fas system in vivo, the mice were quickly killed by liver failure with symptoms similar to fulminant human hepatitis (Ogasawara et al., 1993; Tanaka et al., 1997). Fulminant hepatitis is known to be caused by abnormally activated T cells, and the transformation of hepatocytes with hepatitis B virus or hepatitis C virus causes the up-regulation of Fas expression. These results suggest that under normal circumstances, CTLs recognizing viral antigens expressed on the cell surface of infected hepatocytes are activated through the T cell receptor and kill the hepatocytes via the Fas/FasL system. If this killing process works properly, it benefits the organism. However, when the system is exaggerated, it may lead to fulminant hepatitis. It is possible that other CTL-induced autoimmune diseases such as graft-versus-host disease, AIDS, and insulitis are also mediated by the Fas system.

Various cancer patients produce TNFα in a soluble form, and it works like a cachectin to induce systemic tissue damage. Similarly, the soluble form of FasL was found in the sera of patients with NK lymphoma or large granular lymphocytic leukemia (LGL) of the NK or T cell type (Tanaka et al., 1996). The leukemic cells themselves were found to express functional FasL on their surfaces. These patients often show systemic tissue damage such as hepatitis and neutropenia. Since hepatocytes and neutrophils are particularly sensitive to Fas-mediated apoptosis, it is possible that the systemic tissue damage observed in these patients is due to FasL in their serum or to FasL expressed on the circulating leukemic cells.

Conclusions and Perspectives

Many growth and differentiation factors regulate proliferation and differentiation of mammalian cells during development. So far, three death factors (TNF, FasL, and TRAIL) and four death factor receptors (Fas, TNFR1, DR3/Wsl-1, and CAR1) have been identified. Loss-of-function mutations in the Fas system, *lpr* and *gld* mice, illustrated the importance of this death factor system in maintaining mammalian homeostasis, specifically in the life and death of lymphocytes. It is possible that many more death factor and receptor systems that regulate apoptosis in a tissue-specific manner will be found in the future. Growth and differentiation signals are mediated by the phosphorylation and dephosphorylation of proteins, as well as by small second-messenger molecules such as cAMP and phosphatidyl inositol. These signals are reversible in most cases. On the other hand, the apoptosis signal triggered by death factors is irreversible; that is, a protease cascade is activated by the death signal, and the proteases cleave various cellular

components, which leads to morphological changes of the cells and nuclei that are typical for apoptosis. Since other apoptosis-inducing agents also activate caspase family proteases, their signal transduction system may be similar or identical. However, the apoptotic system in mammals seems to be more complicated and sophisticated than that in C. elegans. Instead of the single ICE/CED-3 and the single Bcl-2/CED-9 in C. elegans, the mammalian genome carries at least ten members of the caspase family and nine members of the Bcl-2 family. Whether they are just redundant or have different roles remains to be examined. Biochemical analysis of each family member and establishment of mice lacking each member will clarify these points. Both TNF and FasL induce apoptosis. However, since TNF can induce other signals, such as activation of NF-κB, it was thought likely that the signaling pathway through the TNF receptor would be more complicated. In fact, identification of the molecules involved in TNF and Fas signaling indicates that the Fas-mediated signal is simpler than that of the TNF receptor. The TNF receptor shares a signal cascade with Fas in one apoptotic pathway, but it also activates additional signaling pathways including one that activates a survival signal. It will be interesting to examine what kinds of survival genes are activated by NF-κB and how these molecules inhibit apoptosis. Identification of these survival genes may provide clues as to why some tumor cells are resistant to various apoptosis-inducing agents including FasL, TNF, and anti-cancer drugs.

As described above, the Fas death factor system is a double-edged sword. If this system is properly regulated, it is useful for down-regulating the immune reaction and for removing virally infected as well as cancerous cells; but, if this system is exaggerated, it can cause tissue destruction. How can modulation of this system be applied to human diseases? The first obvious application is the killing of tumor cells, since some cancer cells, particularly some lymphoid tumors, express functional Fas. However, since the systemic treatment of patients with FasL will cause deleterious side effects, methods of local administration and/or proper targeting of FasL to the tumor should be devised. FasL can also be used as an immune-suppressive agent. As discussed above, the rejection of transplants is mediated by activated T cells. If a transplanted tissue is engineered to express FasL or is cotransplanted with FasL-expressing cells, the transplant may be tolerated. The other application of this system is to block FasL-induced tissue destruction. If Fas is shown to play a role in human diseases such as fulminant hepatitis, AIDS, and other diseases involving CTL-induced tissue destruction, then neutralizing antibodies against Fas or FasL, or other inhibitors of Fas-mediated apoptosis, would have potential as therapeutic agents.

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Note Added in Proof

The paper cited as Rodriguez et al. (1996a) has been published: Rodriguez, I., Matsuura, K., Ody, C., Nagata, S., and Vassalli, P. (1996a). Systemic injection of a tripeptide inhibits the intracellular activation of CPP32-like proteases in vivo and fully protects mice against Fas-mediated fulminant liver destruction and death. J. Exp. Med. *184*, 2067–2072.